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The level of the endoplasmic reticulum stress chaperone protein, binding immunoglobulin protein (BiP), decreases following successful tuberculosis treatment

Bongani Motaung, Gerhard Walzl, Andre G. Loxton*

DST/NRF Centre of Excellence for Biomedical Tuberculosis Research, South African Medical Research Council Centre for Tuberculosis Research, Division of Molecular Biology and Human Genetics, Faculty of Medicine and Health Sciences, Stellenbosch University, PO Box 241, Cape Town 8000 South Africa

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ABSTRACT

An increased *Mycobacterium tuberculosis* burden inside the host leads to higher demand of response proteins. This in turn results in metabolic shift and cellular stress, which is caused by the accumulation and trafficking of these proteins within the endoplasmic reticulum (ER). To resolve this, cells trigger the unfolded protein response (UPR), which is mainly mediated by binding immunoglobulin protein (BiP)/glucose-regulated protein 78 (GRP78) chaperone, and this in turn upregulates its transcription. This chaperone protein facilitates proper protein folding within the ER; however, it can also be passively secreted into the extracellular environment or be expressed on cell surfaces attached to anchor proteins and transmembrane proteins. This notion has been shown in studies on chronic inflammation, including cancer and arthritis, with the detection of BiP-specific antibodies from different sample types. The present study analysed secreted BiP from plasma samples collected from healthy participants and patients with newly diagnosed tuberculosis (TBdx), seen over the course of TB treatment at week 1 (W1), month 2 (M2), and month 6 (M6). The results revealed that during the initial TB disease and treatment period, cells are subjected to stress conditions resulting in metabolic shifts, which lead to the secretion of the intracellular UPR-mediating chaperone protein, BiP. This was indicated by mean differences between TBdx (mean 40.88 ng/ml) and W1 (68.57 ng/ml) in the TB participant groups. However, no difference was observed between the healthy group (mean 42.64 ng/ml) and TBdx group (mean 40.88 ng/ml). Analysis of paired time-point visits revealed increased BiP secretion during early TB treatment. The detection of BiP in plasma samples was found to decrease after successful TB treatment to levels comparable to those in the healthy controls. Evaluation of BiP levels in larger TB treatment studies may lead to the identification of a new target for early TB diagnosis and host-directed immunotherapy.

Introduction

Binding immunoglobulin protein (BiP), also referred to as glucose-regulated protein 78 (GRP78), is an endoplasmic reticulum (ER) chaperone protein that plays a crucial role in protein synthesis by mediating proper protein folding through binding with polypeptide structures entering the ER lumen (Maddalo et al., 2012; Morris et al., 1997). Although BiP interacts with peptides, its function is not required for the extracellular secretion of proteins. This chaperone protein resides in the ER lumen, in an inactive state attached to three membrane transducer proteins: inositol-requiring enzyme 1 (IRE1), protein kinase R-like endoplasmic reticulum kinase (PERK), and activating transcription factor 6 (ATF-6). Activation of this chaperone occurs during conditions that cause cellular stress (Gass et al., 2002), specifically on ER, triggering the unfolded protein response (UPR), which has been reported as a cell survival mechanism.

During the UPR, a series of events may occur, including possible termination of transcription and translation, destruction of unfolded or misfolded proteins through ubiquitination, and upregulation of GRP78/BiP expression (Rao and Bredesen, 2004). Although this response is chiefly mediated by BiP/GRP78, it also requires the activity of the three signal transducers localized in the ER membrane (IRE1, PERK, and ATF-6), which phosphorylates cytosol kinases, and these metabolic pathways determine the fate of the cell during the UPR by either promoting cell survival or apoptosis.

* Corresponding author.
E-mail address: GL2@sun.ac.za (A.G. Loxton).

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During the UPR, BiP/GRP78 transcription is upregulated and this is mediated by the transcription factor YY1, which binds to the promoter region of the BiP gene. This transcription factor also acts as a cofactor for the activation of ATF-6, which is reported to be essential during the initiation of the ER stress response (Baumeister et al., 2005). BiP upregulation has also been shown to be influenced by the activity of some proinflammatory cytokines, particularly tumour necrosis factor alpha (TNF-α) and interleukin 1β (IL-1β) (Yoo et al., 2012).

Research studies have also shown that during chronic inflammation and prolonged ER stress, BiP can translocate to other cell compartments such as the cell membrane where it can become attached to transmembrane proteins or glycosylphophatidylinositol (GPI) proteins. A study by Tsai et al. (2015) suggested that BiP can also escape the ER lumen to other cell compartments attached to recently synthesized proteins. This could imply that during the UPR, BiP may be secreted into the extracellular fluids through these processes, and it was shown that its substrate binding activity was important in this regard (Tsai et al., 2015).

Previous studies have reported an association between cell-free BiP and disease exacerbation; however these studies focused on chronic diseases such as cancer and autoimmune disease (Bläss et al., 2001; Bodman-Smith et al., 2003; Zhang et al., 2010). Moreover, the mechanisms involved in the secretion of this chaperone into the extracellular fluids are not clearly defined and it is also thought to escape during membrane repair by ER vesicles (Zhang et al., 2010). In an extracellular phase, this chaperone has been shown to act as an autoantigen on immune cells and leads to metabolic shifts that affect cell function (Bodman-Smith et al., 2003). The presence of BiP in extracellular fluids has been shown in different sample types including serum, plasma, and synovial fluid.

Varying disease outcomes have been associated with extracellular BiP, which show it to be both beneficial and detrimental. It has been reported that this chaperone protein is overexpressed in rheumatoid arthritis (RA) through the detection of autoantibodies secreted specifically against it. During RA, extracellular BiP has been reported to upregulate anti-inflammatory responses and this has previously been reported to be beneficial in resolving inflammation (Corrigall et al., 2004). Contradictory results have also been reported, with extracellular BiP being shown to enhance the progression of RA in synoviocytes by promoting the proliferation of synoviocytes, which in turn enhance angiogenesis (Yoo et al., 2012). Additionally, the presence of this chaperone protein in the extracellular phase has been reported to enhance outcome of RA by affecting the activity of cytotoxic T-cells (Bläss et al., 2001). Similarly, BiP pathogenesis has been reported during cancer inflammation and it has been suggested that the downregulation of BiP synthesis and activity could be beneficial in resolving cancer conditions by restricting the synthesis of vital proteins required by the cancer cells (Schwarze and Rangnekar, 2010).

Immunological response modifications associated with this chaperone protein have led to interest in research evaluating its secretion during the anti-tuberculosis (TB) treatment period in infected patients. Previous immunological response studies have highlighted the dysfunctionality within the B-cell subset in response to TB and this may in part be brought into play by the extracellular matrix. Extracellular BiP binding may lead to intracellular metabolic shifts that affect the expression of both soluble proteins and cell surface proteins.

Materials and methods

Ethics statement

Ethical approval was obtained from the Health Research Ethics Committee of Stellenbosch University (N05/11/187) and the City of Cape Town City Health. The study was conducted in accordance with the Declaration of Helsinki and International Conference on Harmonisation guidelines. Written informed consent was obtained from all study participants.

Sample description

Blood samples were collected from participants recruited in the Sarepta area of Cape Town in the Western Cape region of South Africa. Participants were of both sexes and were aged between 18 and 64 years. Plasma samples were collected from a total of 81 participants (Figure 1), of whom 42 were male and 39 were female. Collected samples were placed into two groups: those from healthy controls (32 participants) who had no indication of *Mycobacterium tuberculosis* infection based on sputum culture, X-ray, and Quantiferon interferon-gamma release assay (IGRA); and those from patients with a diagnosis of active TB (TBdx, disease group at diagnosis; 29 participants) who had a positive sputum culture, X-ray, and signs and symptoms suggestive of TB disease. TB patients were started on treatment and were followed up at three time-points; week 1 (W1), month 2 (M2), and month 6 (M6). Collected plasma samples were then stored at −80 °C until analysis.

Enzyme-linked immunosorbent assay (ELISA) analysis

Collected plasma samples were recovered from the biorepository and analysed by ELISA. The samples included those from the healthy controls ($n = 32$) and the TB disease group ($n = 29$ at baseline (TB diagnosis); $n = 8$ for the W1 follow-up time-point; $n = 7$ for the M2 follow-up time-point; $n = 19$ for the M6 follow-up visit); in addition, 20 new participants were recruited at the M6 time-point.

Plasma samples were completely thawed at room temperature before ELISA analysis. They were then centrifuged at $1000 \times g$ for 5 min to pellet any particulate material that might interfere with analyte binding. The Human GRP78 ELISA kit from MyBioSource (MBS2533401; San Diego, USA), which has a minimum sensitivity of 0.375 ng/ml and a detection range of 0.625–40 ng/ml, was used as per the manufacturer's instructions. The kit is specified to have no cross-reactivity or interference between analogues and human GRP78. ELISA plates were read using a Bio-Rad iMark Microplate reader (California, USA) at a recommended wavelength of 450 nm.

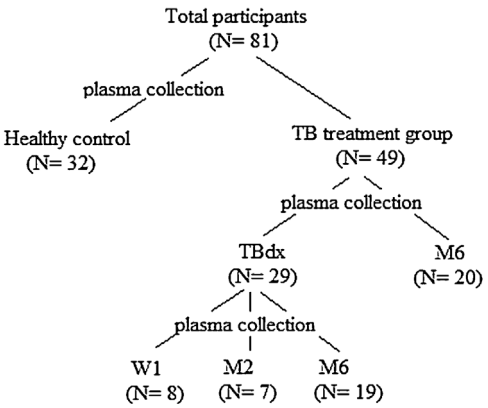

Figure 1. Plasma sample collection at specific time points. *N* = total number, TB = tuberculosis, TBdx = TB diagnosis, W1 = week 1, M2 = month 2, and M6 = month 6.

Statistical analysis

The optical density (OD) readings obtained were analysed using GraphPad PRISM 7 for Windows (GraphPad Software, La Jolla, California, USA; www.graphpad.com). BiP concentrations were analysed for differential expression between groups using one-way non-parametric analysis of variance (ANOVA), the Mann–Whitney U-test, and the unpaired *t*-test.

Results

BiP levels remain unchanged at diagnosis of active TB compared to healthy controls

Figure 2 shows that although BiP is an ER resident chaperone protein, it can be secreted into the plasma in small quantities even in healthy individuals. There was a statistically significant difference in BiP secretion between the participant groups ($p = 0.0270$), with the healthy group (mean 42.64 ng/ml) and the TB patients at diagnosis (TBdx) (mean 40.88 ng/ml) having comparable mean expression. BiP secretion was upregulated during early TB treatment (W1), showing the highest mean of 68.57 ng/ml. Furthermore, the detection of BiP in the TB groups decreased gradually over the course of TB treatment, approaching the levels of the healthy group and the TBdx baseline group (Figure 2). In the second month of TB treatment (M2), BiP detection was found to decrease (mean 60.92 ng/ml) and there was a further decrease by month 6 (M6), as indicated by mean value of 37.42 ng/ml. In contrast, no statistically significant difference was observed between the healthy, TBdx, M2, and M6 groups.

Effect of TB treatment on follow-up participants between TBdx and W1

BiP upregulation was further illustrated in TB treatment follow-up participant pairs (Figure 3, Table 1), which indicated direct upregulation of this chaperone from respective individuals with a detection range between 30.60 ng/ml and 65.57 ng/ml for TBdx and between 60.18 ng/ml and 86.77 ng/ml for W1 anti-TB treatment. This showed a significant difference ($p = 0.0078$) in BiP secretion between these two time-point visits.

BiP levels remain unchanged from week 1 of TB treatment until the end of therapy

The participant follow-up visit at month 2 of anti-TB treatment showed no significant change in extracellular BiP detection (Figure 4) with their respective diagnosis time point ($p = 0.1563$)

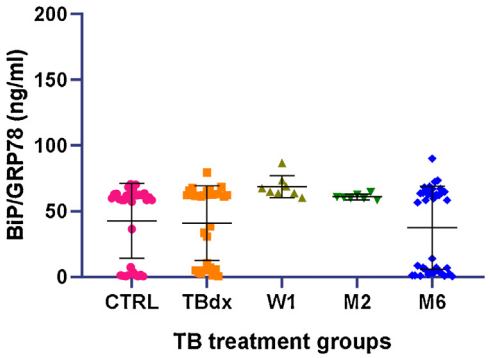

Figure 2. Detection of secreted binding immunoglobulin protein (BiP) during TB treatment and healthy states. Statistical difference analysis through non-parametric one-way ANOVA, $p = 0.0270$. CTRL = healthy group, TBdx = TB treatment group at diagnosis, W1 = week 1 treatment group, M2 = month 2 treatment group, and M6 = month 6 treatment group; error bars show the mean with standard deviation.

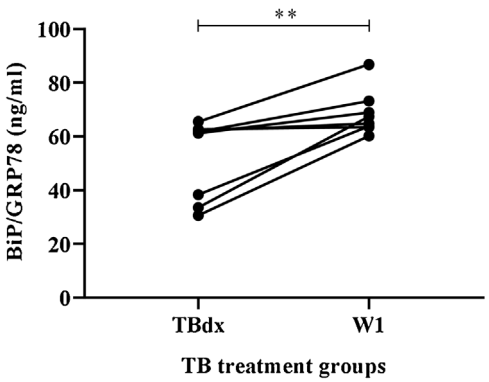

Figure 3. Increased levels of binding immunoglobulin protein (BiP) following 1 week of TB treatment. Sample pairs analysed for changes in BiP secretion 1 week after the start of TB treatment. Statistical difference assessed through non-parametric paired *t*-test (Wilcoxon matched pairs test), $p = 0.0078$; $^{**}p < 0.01$.

and a detection range between 58.41 ng/ml and 64.80 ng/ml, as shown in Table 1. BiP detection at the end of anti-TB treatment (month 6) indicated a detection range between 56.64 ng/ml and 73.45 ng/ml; this showed no significant difference with BiP detection at TBdx ($p = 0.4800$) (Table 1).

Discussion

This study provides evidence that during TB disease treatment, the UPR is activated, leading to upregulation of intracellular mediators such as BiP, which in turn escape into the extracellular phase. Activation of the UPR is regarded as a survival mechanism used by cells to cope with a high demand of response proteins during inflammation (Lin et al., 2007).

M. tuberculosis infection is classified into different infection stages, with latent TB infection (LTBI) representing the early stage of infection, in which the bacterium is thought to be contained (Flynn and Chan, 2001). During this time, immune cells are subjected to minimal metabolic shift, resulting in minimal stress response activation; this is evident in the present study results by the non-significant different in BiP secretion levels between healthy participants and those diagnosed as positive for *M. tuberculosis* infection via sputum culture, IGRA, and X-ray results.

This finding is comparable to those reported in other studies performed on cancer and arthritis, which have shown that during prolonged stress conditions, cells activate the UPR response resulting in the secretion of intracellular ER chaperone proteins, amongst which is BiP (Bodman-Smith et al., 2003; Zhang et al., 2005). Although the mechanisms involved in this are not fully elucidated, it has been suggested that the cells elude these intracellular components through passive mechanisms during ER vesicle translocation, or they can also be released in conjunction with their protein substrates (Tsai et al., 2015).

M. tuberculosis has been shown to be able to manipulate and avoid the innate immune responses and multiply within the host (Raghuvanshi et al., 2010; Sia et al., 2015). This leads to an increased bacterial burden, which may eventually activate the UPR due to the high demand of immunoglobulins and metabolic shift. This then leads to the secretion of these mediators into the extracellular phase, where they affect the disease outcome.

However, pharmacological agents have also been implicated in activating the UPR (Srinivasan et al., 2005). This may suggest that TB treatment regimens also participate in the upregulation of the UPR, as they need to be metabolized by cells, mainly liver cells, into inactive forms before being eliminated from the body. This results in metabolic shifts within the cells, which force them to upregulate the transcription and synthesis of specific protein compounds to

Table 1
TB treatment follow-up participants.

Variable	Treatment groups	Number[a]	Mean levels (ng/ml)	SD	*p*-Value	95% CI for the mean		Minimum (ng/ml)	Maximum (ng/ml)
						Lower bound	Upper bound		
BiP	TBdx	8	52	14.95	0.0078	36.5	64.5	30.60	65.57
	Week 1[b]	8	67.91	8.78		60.57	75.25	60.18	86.77
	TBdx	7	50.06	15.03	0.1563	36.16	63.96	30.60	62.74
	Month 2[b]	7	60.92	2.10		58.98	62.86	58.41	64.80
	TBdx	19	59.50	12.90	0.4800	52.87	66.13	30.60	79.36
	Month 6[b]	19	64.59	4.43		62.31	66.87	56.64	73.45

TB, tuberculosis; SD, standard deviation; CI, confidence interval; BiP, binding immunoglobulin protein; TBdx, TB diagnosis.
[a] Number of participants.
[b] Week 1, month 2, and month 6 of TB treatment.

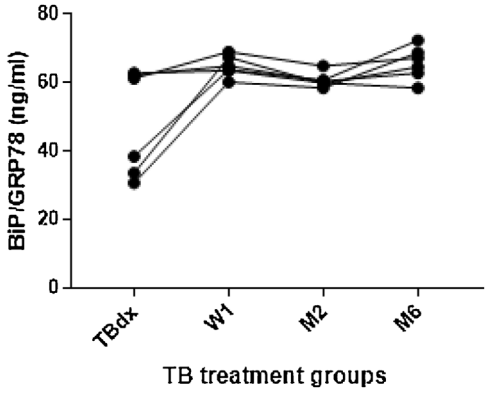

Figure 4. Paired time-point visits during TB treatment revealed increased secretion of binding immunoglobulin protein (BiP) by TB treatment group. Statistical difference assessed through non-parametric paired Friedman test, $p = 0.0057$.

mitigate the condition, among which is BiP. The latter phenomenon could be correlated with the high secretion levels (Figure 2) of this stress response protein during the initial treatment period, which is also characterized by a sharp decline in bacterial numbers. Even though BiP is upregulated during the initial metabolic shifts (Bodman-Smith et al., 2004), transcription and protein synthesis becomes stabilized with unchanged conditions or declines with resolved inflammation. Theoretically, during the TB treatment period, the decrease in bacterial burden over time should result in a decreased metabolic demand from activated immune cells; this correlates with the study findings, where extracellular BiP was shown to stabilize and gradually decrease after week 1 of TB treatment. Similarly, the switch of TB drugs after month 2 (from four drugs to two) and decreased bacterial burden do not facilitate further upregulation of the UPR; furthermore, in contrast to the other drugs in the TB regimen, rifampicin is not metabolized in the body, suggesting an even lower protein demand.

Although activation of the UPR is considered a cell survival mechanism, it has also been shown that a prolonged response triggers cell death through apoptotic pathways by upregulation of the transcription factor C/EBP homologous protein (CHOP) (Mozos et al., 2011). The latter can be beneficial for infection control, since TB disease is caused by an intracellular pathogen and inducing apoptosis results in cell death from within by limiting metabolite flux, which is vital for *M. tuberculosis* survival. Apoptotic pathways are known to prevent early cell membrane rupture, thus limiting the spread of *M. tuberculosis* to uninfected cells. This is the opposite of necrotic pathways, which are suggested to be utilized by the pathogen in question to infect and reside in other immune cells.

Upregulation of BiP in the cellular microenvironment has been shown to induce an anti-inflammatory response in immune cells, either by upregulation of interleukin 10 (IL-10) synthesis (Yoshida et al., 2011) or upregulation of surface markers involved in programmed cell death (Tang et al., 2016).

This study evaluating the utility of secreted BiP over the course of TB treatment must be seen as an preliminary investigation, as the number of participants with active TB was relatively small. BiP levels were detected with standard methods; however protein degradation might have occurred over time, resulting in lower levels of this biomarker. Future studies must evaluate BiP levels in a larger collection of fresh plasma and serum samples from participants at different stages of *M. tuberculosis* infection.

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Ethical approval

Ethical approval was obtained from the Health Research Ethics Committee of Stellenbosch University (N05/11/187) and the City of Cape Town City Health. The study was conducted in accordance with the Declaration of Helsinki and International Conference on Harmonisation guidelines. Written informed consent was obtained from all study participants.

Conflict of interest

The authors declare no conflict of interest.

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